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The energetic network of hotspot residues between Cdc25B phosphatase and its protein substrate

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Summary

We have investigated the functional network of hotspot residues at the remote docking site of two cell cycle regulators, namely Cdc25B phosphatase and its native protein substrate Cdk2-pTpY/CycA. Specifically, we have studied the roles of energetically important residues (Arg488, Arg492, Tyr497 on Cdc25B and Asp206 and Asp210 on Cdk2-pTpY/CycA) by generating a diverse set of substitutions and performing double- and triple mutant cycle analyses. This transient protein-protein interaction is particularly well-suited for this mutagenic approach because various control experiments ensure that the effect of each mutation is limited to the interaction of interest. We find binary coupling energies for ion pairs and hydrogen bonds ranging from 0.7 to 3.9 kcal/mol and ternary coupling energies of 1.9 and 2.8 kcal/mol. Overall our biochemical analyses are in good agreement with the docked structure of the complex and suggest the following roles for the individual hotspot residues on Cdc25B. The most important contributor, Arg492, forms a specific and tight bidentate interaction with Asp206 and a weaker interaction with Asp210 that cannot be replaced by a Lys. Although Tyr497 does not directly participate in this ionic network, it is important for buttressing Arg492 using both its hydrophobic (aromatic ring) and hydrophilic characteristics (hydrogen bonding). Arg488 participates less specifically in the electrostatic network with Asp206 and Asp210 of the protein substrate as it can partially be replaced by Lys. Our data provide insight how a cluster of residues in a docking site remote from the site of the chemical reaction can bring about efficient and specific substrate recognition.

Keywords

protein-protein interactions; coupling energy; mutant cycle analysis; Cdc25 phosphatase; Cdk2

Introduction

The details of protein association and binding are best understood by elucidating the functional networks of residues between interacting proteins. High resolution structures are valuable assets for functional studies and usually serve as starting points for exploring energetics at protein interfaces. However, predicting the energetic contributions of individual interfacial residues based solely on structural data can be challenging. A typical protein-protein interface encompasses $\geq 1600 \text{ \AA}^2$ or ≥ 30 surface residues, yet the majority of the interaction energy between two proteins is governed in large part by just a few (5-10%) so-called hotspot residues 1; 2; 3. Even though some residues (e.g. Asp, Glu, and Arg) are slightly enriched in such hotspots, the residue composition and interface topologies hotspot sites are rather unique for individual systems. Therefore, energetically important interactions at protein interfaces are most often elucidated only by biochemical analyses.

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One of the most commonly used techniques to identify hotspot residues and their roles at protein interfaces is site-directed mutagenesis, wherein a residue of interest is mutated such that the original functional group is removed (i.e. alanine scanning). Generating single deleterious mutations is the first and essential step for detailed functional studies. However, this technique by itself has some significant drawbacks. Even if control experiments ensure that the outcome of each mutation is limited to the parameter of interest (k_{cat} , K_m , K_d , etc.), single mutant analysis cannot elucidate pair-wise interactions across a protein-protein interface. Moreover, such an approach often neglects the interconnectivity of residues intrinsic to protein-protein interactions^{4; 5}. Because observed phenotypes upon mutation can arise from cooperative networks of residues, rather than direct contacts, any interpretations made regarding the role of residues based solely on single mutant analysis must be made cautiously.

Functional interactions across protein-protein interfaces can be explored more thoroughly by mutant cycle analyses with which one can quantitatively assess energetic relationships between two or more residues^{6; 7; 8; 9}. Double mutant cycle analysis, for example, derives coupling interactions between two residues by comparing the effects of mutating individual residues vs. the outcome of simultaneously mutating both residues. When two residues do not interact with each other, in other words, when the damaging effect of the mutations is mutually exclusive, the outcome of a double mutation is essentially additive. On the other hand, when the two residues are involved in an energetic interaction while promoting the same step during binding, the outcome of double mutation deviates significantly from the theoretical sum of the damage caused by the individual mutations. In the analysis by double mutant cycles, this non-additivity can be quantitatively described by the coupling energy (cooperativity) of two residues. Furthermore, although coupling energy is strictly a thermodynamic term, a pair of interacting hotspot residues located in close proximity ($< 5\text{\AA}$) generally displays significant cooperativity. Thus, analysis by double mutant cycles has also been used to obtain approximate spatial relationships among energetically important residues at protein-protein interfaces^{10; 11; 12; 13}. However, interpreting double mutant cycle data can be tricky as the coupling energy includes indirect interactions arising from a larger network of surrounding residues¹⁴. These networks can be further explored quantitatively by triple mutant cycle analysis where the coupling energy between a pair of residues is assessed in the presence or absence of a nearby residue¹⁵. Although it is practically impossible to mutate a large number of residues in higher order mutant cycles without altering the overall stability and integrity of a protein, with proper controls one can readily identify and study in detail functionally important contacts and networks for a given protein-protein interface.

We have been studying the transient protein-protein interactions between Cdc25 phosphatases and their protein substrates. Cdc25s are dual specificity phosphatases involved not only in regulation of the normal eukaryotic cell cycle, but also in the checkpoint response^{16; 17; 18}. The role of the Cdc25s is to remove two inhibitory phosphates located near the active sites of the Cdk/Cyclin complexes, thereby activating the kinase activity of the Cdk/Cyclins and promoting cell cycle progression. The critical involvement of Cdc25 phosphatases in the regulation of the cell cycle is exemplified by the overexpression of two Cdc25 isoforms (Cdc25A and Cdc25B) in a number of cancers. Understanding the details of the interactions between the Cdc25s and their Cdk/Cyclin substrates is expected to facilitate the development of small molecule inhibitors that specifically target this interaction. For the Cdc25s, the majority of the binding interaction with protein substrate resides outside the active site, as indicated by the six-orders of magnitude preference for protein substrate vs. phospho-peptide substrates that contain the same primary sequence¹⁹. For Cdc25B we have identified this protein docking site and its conserved hotspot residues, $\geq 20\text{\AA}$ away from the active site (Arg488, Arg492, Tyr497) (Figure 1)^{20; 21}. We have used computational protein docking and molecular dynamics to propose a docked orientation for the Cdc25B enzyme – Cdk2-pTpY/CycA substrate and to identify the corresponding hotspot residues on the protein

substrate (Asp206 and Asp210 of the Cdk2 subunit) (Figure 1). We have validated our proposed docked orientation by crystallography, selected double mutant cycle analysis, and swapping of two charged hotspot residues²¹.

In this report we delve deeper into the details of the functional interactions among these hotspot residues using several mutagenesis techniques. First, we address the chemical functionality of the hotspot residues of Cdc25B by incorporating alternative amino acid substitutions at each position. We then expand the analysis by double mutant cycles with these alternative mutants to decipher the nature of the interactions between the hotspot residues on the enzyme and the substrate. Furthermore, we perform triple mutant cycles to probe the interconnectivity of the observed coupling energies in this network of hotspot residues.

RESULTS

Experimental rationale and control experiments

The interaction between Cdc25B phosphatase and its protein substrate serves as a particularly good system for elucidating the energetics of transient protein-protein interactions by mutagenesis and mutant cycle analysis. Our assay measures the Cdc25B-catalyzed phosphorolysis of pThr14 from the bis-phosphorylated Cdk2-pTpY/CycA substrate. Assays performed under k_{cat}/K_m conditions reflect the rate of E*S complex formation for wild-type enzyme and substrate as well as all mutants investigated to date (manuscript in preparation). The Cdc25B – Cdk2-pTpY/CycA system also allows for sensitive controls to ensure that hotspot mutations do not drastically perturb the general structure or other selected functions of the enzyme or its substrate. For Cdc25B, given that the hotspot region is located remote from the active site, we can test the activity of each mutant with small molecule substrates such as *p*-nitrophenyl phosphate (pNPP) and 3-*O*-methyl fluorescein phosphate (mFP). For both substrates, the catalytic cysteine must exist as a thiolate within the active site pocket²². The reaction with pNPP probes substrate association (k_{cat}/K_m) and phospho-enzyme formation (k_{cat}) whereas the reaction with mFP (k_{cat}) probes hydrolysis of the phospho-enzyme intermediate²³. For Cdk2/CycA, phosphorylation by the Myt1 kinase during substrate preparation allows for accurate quantitation of intact protein substrate. Also, assay of the Cdk2/CycA complex as a kinase with its histone substrate can further ensure the integrity of the substrate protein^{19; 20; 21}. Such a convenient dissection of functions has not proven feasible for other protein-protein interaction systems (e.g. 14).

Alternative hotspot substitutions

We first consider the chemical nature of the interactions provided by the three hotspot residues of Cdc25B (Arg488, Arg492, and Tyr497) by incorporating alternative amino acid substitutions. The original hotspot mutations (R488L, R492L, and Y497A) were chosen to minimize perturbation of the protein structure while maximizing any effects due to loss of functional groups. By introducing more conservative substitutions we can assess the role of each component of the original residues. For example, Arg can be substituted by Lys and Tyr can be substituted by Phe. As for the original hotspot mutants of Cdc25B, we first verified that each of the mutations did not perturb the essential chemistry at the active site by testing their activity against small molecule substrates (Table 1). Although certain mutants (e.g. R492L-Y497A in particular) displayed kinetic parameters somewhat different than wild-type in either the first half-reaction to form the phospho-enzyme intermediate and/or the second half-reaction, hydrolysis of this intermediate, these differences were small compared to the significantly altered rates observed with protein substrate. Also, most observed changes in activity with small molecule substrates led to increased activity, not decreased, as seen with the protein substrate.

A conservative Lys replacement at Arg488 (R488K) showed a significant recovery (15-fold in $k_{\text{cat}}/K_{\text{m}}$) toward protein substrate compared to the Leu substitution (R488L), while R488A showed essentially identical activity as R488L (Table 1). At this site the introduction of a positive charge is sufficient to rescue part of the original function of the Arg, suggesting a significant contribution of electrostatic forces. The lack of complete restoration in the R488K mutant is probably because the location of the charge is only proximal, not precise. For Tyr497, the presence of the aromatic ring and the hydrogen bond seem equally important at the interface as the Y497F mutant showed a 10-fold decrease in $k_{\text{cat}}/K_{\text{m}}$ compared to a 200-fold decrease for the Y497A mutant (Table 1). Not surprisingly, the correct placement of this hydrogen bond is critical as the Y497T substitution resulted in an essentially identical phenotype to Y497A (Table 1). Alternative substitutions at Arg492 showed an unexpected range of activities (Table 1). To our surprise, the lysine substitution (R492K) resulted in an even more dramatic decrease in $k_{\text{cat}}/K_{\text{m}}$ specifically towards protein substrate when compared to R492L (4600-fold vs. 200-fold). More interestingly the observed $k_{\text{cat}}/K_{\text{m}}$ of R492K is comparable and in fact marginally lower than that seen for the R492L-Y497A double mutant (see below). Furthermore, although we expected the Ala substitution (R492A) to have the most detrimental effect, the R492A actually showed a much smaller decrease in $k_{\text{cat}}/K_{\text{m}}$ than its R492L counterpart (40-fold vs. 200-fold). We suggest a partial rescue by the adjacent Arg488, wherein this residue can substitute somewhat for Arg492 in the R492A mutant, but not in the R492K mutant. Taken together, these observations for substitution of Arg492 suggest that the interaction provided by this residue is mostly short-range and specific (ionic hydrogen bond) rather than simple electrostatic charge complementary across the interface.

Double mutant cycles

Using these alternative substitutions of the hotspot residues, we next performed analysis by double mutant cycles with the two Asp hotspot residues on the substrate (Asp206 and Asp210 on Cdk2, Figure 2). In our initial double mutant cycle analysis²¹ we used mutant enzymes/substrates wherein the interactions between the proposed hotspot residues were almost completely abrogated and the observed pair-wise coupling energies ($\Delta\Delta G_{\text{int}}$) were maximized. However, if more conservative mutations (i.e. R488L vs. R488K) can partially re-establish interactions between two hotspot residues (or a network of interactions including the mutated pair), the $\Delta\Delta G_{\text{int}}$ obtained from double mutant cycle analysis is expected to be less than the original mutant pair¹⁴. Therefore, we can quantitatively determine the contribution of key functional groups to the observed cooperativity by comparing the $\Delta\Delta G_{\text{int}}$ s of the two pairs of double mutants. More importantly, we can obtain some clues regarding the nature of energetically important interactions (long-range electrostatics vs. short-range hydrogen bonding). It should be noted that the measure of cooperativity between a pair of residues ($\Delta\Delta G_{\text{int}}$) is independent of the single mutant data ($\Delta\Delta G_1$). For example, even though the Y497A mutant enzyme displayed a significantly higher $\Delta\Delta G_1$ compared to Y497F, the $\Delta\Delta G_{\text{int}}$ s of these two residues against the D210A mutant of the protein substrate were essentially identical (see below). One should also remember that any coupling energy is a complex sum of direct and indirect interactions. Thus, the majority of observed cooperativity for the original double mutant pair may have originated from a network of interactions in which these residues participate. If this is the case, any reduction in coupling energy can arise from recovering (or enhancing) nearby functional interactions, not necessarily from restoring the direct interaction between these two residues (see below and triple mutant cycles). Nevertheless, by comparing the proposed roles of individual components from the docked model with the observed changes in cooperativity, we can further validate our docked model and explore in more detail the energetics of these hotspot residues in protein substrate catalysis.

The observed $\Delta\Delta G_{\text{int}}$ from the R488A-D206A pair was comparable to the cooperativity observed for the R488L-D206A pair (Figure 2). On the other hand, the R488K-D206A pair

displayed a $\Delta\Delta G_{\text{int}}$ of 1.1 kcal/mol, which restores 1.8 kcal/mol compared to the maximum disruption of the R488L-D206A pair (Figure 2). These data suggest that the network of electrostatic forces arising from the charge complementary between Arg488 and Asp206 contribute significantly to the observed cooperativity between these two residues (Figure 1). The incomplete restoration of full activity in the R488K mutation may be due to misplacement of the charge by the different length of Lys or the inability of the Lys substitution to recover a specific short-range interaction. Comparisons between the $\Delta\Delta G_{\text{int}}$ of the R488L-D210A pair and those from the R488A-D210A and R488K-D210A pairs showed the same trends as seen from the interactions with Asp206 (Figure 2), suggesting that electrostatics play a significant role in the interaction between Arg488 and this other Asp also. Taken together, these data are in good agreement with the docked model (Figure 1) where Arg488 contributes most significantly to the energetics at the interface by participating in a network of charge complementary.

For Arg492, double mutant cycles with R492A and D206A showed comparable $\Delta\Delta G_{\text{int}}$ s as seen for the R492L mutant (Figure 2), despite the significantly more favorable $k_{\text{cat}}/K_{\text{m}}$ seen for R492A compared to R492L (Table 1). This observation demonstrates the value of mutant cycle analysis compared to single mutation experiments. Unlike for Arg488, the observed difference between the $\Delta\Delta G_{\text{int}}$ s of R492K vs. R492A towards the D206A mutant was only marginal (3.1 vs. 3.5 kcal/mol, respectively). These data suggest that the cooperativity between Arg492 and Asp206 is established through more specific interactions between the guanidino group and the carboxyl group, rather than through simple charge complementary (Figure 1). For the R492A-D210A pair the $\Delta\Delta G_{\text{int}}$ was essentially identical to both the R492L-D210A and the R492L-D210A pairs (1.5 vs. 1.9 vs. 1.6 kcal/mol, respectively). Overall, these data are in good agreement with the docked model wherein Arg492 and Asp206 establish a specific bidentate hydrogen bond and the cooperativity between Arg492 and Asp210 is likely to arise through an indirect but specific interaction (i.e. water mediated hydrogen bond) (Figure 1).

The Y497F-D206A pair showed a $\Delta\Delta G_{\text{int}}$ of 0.7 kcal/mol, accounting for about half of the observed $\Delta\Delta G_{\text{int}}$ from the Y497A-D206A pair (1.6 kcal/mol). This indicates that the hydrophobic interactions provided by the aromatic ring are an important contributor for the observed cooperativity between these two residues. However, it is unlikely that such significant hydrophobic interactions exist directly between the phenyl ring of Tyr497 and the negatively charged carboxylate of Asp206. On the other hand, Tyr497 and Arg492 form a π -cation packing interaction that bolsters the bidentate ionic hydrogen bond between Arg492 and Asp206. Thus, this observed cooperativity is probably more closely linked to the nearby interactions centered on Asp206 and Arg492 (Figure 1, see triple mutant cycle and Discussion). The other half of the $\Delta\Delta G_{\text{int}}$ can be attributed to the hydroxyl group, which may contribute significantly through either a water-mediated or direct hydrogen bonding interaction. However, according to our docked model (Figure 1) it is unlikely that Tyr497 can establish a direct hydrogen bond with Asp206 (-OH of Tyr497 to O- of Asp206 is $> 7 \text{ \AA}$). Thus this observed cooperativity may originate from either a coordinating water molecule from a hydrogen bonding network of neighboring residues including Arg492 and Asp206 (or Asp210), or as a part of the protective mechanism for Arg492 described above. In contrast to the interactions with Asp206, the observed $\Delta\Delta G_{\text{int}}$ from the Y497F-D210A pair was essentially identical to the Y497A-D210A pair (1.3 vs. 1.5 kcal/mol), suggesting a lack of hydrophobic contribution to the cooperativity between Tyr497 and Asp210. These observations are consistent with our docked model in which the interactions between these two residues are dominated by a hydrogen bond between the hydroxyl group of Tyr497 and the carboxyl group of Asp210 (Figure 1). The $\Delta\Delta G_{\text{int}}$ s seen for the Y497T mutant with both D206A and D210A mutants compared to Y497A (Figure 2) is most likely due to a general disruption of the hotspot site by the β - substitution. Taken together, these data differentiate the functional interactions between Tyr497 and the two Asp residues on the substrate into their hydrophobic and hydrogen bonding contributions.

Triple mutant cycle

To quantitatively evaluate the energetics among these interfacial residues of Cdc25 and Cdk2/CycA more closely, we expand the two-dimensional thermodynamic boxes to three-dimensional cubes to accommodate the triple mutant cycle analyses of R492L-Y497A-D206A and R492L-Y497A-D210A (Figure 3). Ternary coupling energy is the interaction energy of three residues that are cooperatively involved in promoting the same process during binding or catalysis. It is just like the binary coupling energy, yet expanded to include three residues. To perform these analyses, we first needed to determine the pair-wise interaction energy between Arg492 and Tyr497 on Cdc25 by measuring the activity of the double mutant of R492L-Y497A toward wild-type protein substrate. As expected by their close proximity, a significant $\Delta\Delta G_{\text{int}}$ of 2.1 kcal/mol was observed from the intra-double mutant cycle of these two residues (Table 1, Fig. 4A, Fig. 5A, Table 2). This non-additive term demonstrates that these two residues contribute to the same process of protein substrate recognition by Cdc25, and should not be interpreted as the amount of π -cation interaction between these two residues.

The overall cooperativity of Arg492 and Tyr497 with Asp206 and Asp210 was obtained by triple mutant cycle analysis (Fig. 4, 5, Table 2). Ternary coupling energies of 2.8 kcal/mol and 1.9 kcal/mol were observed for R492L-Y497A-D206A and R492L-Y497A-D210A, respectively. These ternary coupling energies imply cooperativity among these hotspot residues during protein substrate recognition. In other words, the damaging effects of any single or double mutants of these residues are at least partially offset by the ternary coupling energy of the network of these three residues. These ternary coupling energies are higher than those observed for a cluster of residues in TEM-1- β -lactamase and its inhibitor BLIP (0.86 kcal/mol)¹⁵. This increased coupling may arise from the bidentate hydrogen bond between Arg492 and Asp206 for our system being more dependent on exact orientation within the cluster compared to the two individual salt bridges that Asp49 makes with Lys234 and Arg243 in the Schreiber system.

We next evaluate the cooperativity of two residues in the presence or absence of the third residue more closely. The contribution of the third residue to the observed cooperativity of two residues can be quantitatively determined by comparing the $\Delta\Delta G_{\text{int}}$ s of two double mutant cycles in the presence or absence of the third residue (Figures 4 and 5, Table 2). For example, panel A of Figure 4 describes the cooperativity between Arg492 and Tyr497 in the presence of Asp206, whereas panel B of Figure 4 shows the cooperativity between these two residues in the absence of Asp206. We first consider the cooperativity among Arg492, Tyr497, and Asp206 (Figure 4). Both our docked model and double mutant cycles have suggested that the cooperativity seen from Tyr497 in protein substrate catalysis is likely to originate through networking with neighboring interactions. In good agreement with these earlier observations, the cooperativity between Arg492 and Tyr497 from the double mutant analysis is largely dependent on the presence of Asp206 since eliminating Asp206 makes the coupling between Arg492 and Tyr497 unfavorable (−0.8 kcal/mol, Figure 4 panel A vs. B). Our earlier observations also suggested that Arg492 and Asp206 form a direct bidentate ionic hydrogen bond interaction (Figure 1 and 2). Consistent with these observations, the presence of Tyr497, although clearly enhancing the interaction between these residues, is not absolutely required for the coupling interaction between Arg492 and Asp206 since the $\Delta\Delta G_{\text{int}}$ is still a favorable positive value even in the absence of Tyr497 (1.1 kcal/mol, Figure 4 panel C vs. D). On the other hand, the observed cooperativity between Tyr497 and Asp206 is highly dependent on the presence of Arg492 since removing Arg492 results in a large negative $\Delta\Delta G_{\text{int}}$ (−1.3 kcal/mol, Figure 4 panel E vs. F). This is again consistent with our docked model and double mutant cycle analysis, which suggest that the observed coupling energy between these two residues arises from participating in the interaction between Arg492 and Asp206 (i.e. Tyr497 protecting the partially buried salt bridge of Arg492-Asp206). Nevertheless, any unfavorable individual

pair-wise interactions are converted to favorable cooperative interactions via the overall ternary coupling of these residues (~ 2.8 kcal/mol).

We next consider the cooperativity between Arg492, Tyr497 and Asp210 (Figure 5, Table 2). As expected by its location in the docked model, the contribution of Asp210 to the observed cooperativity between Arg492 and Tyr497 is moderate and not as significant as seen for Asp206. The $\Delta\Delta G_{\text{int}}$ between Arg492 and Tyr497 becomes almost neutral (zero) in the absence of Asp210, but still remains favorable (Figure 5 panel A vs. B). The absence of Tyr497 also does not impose much negative effect on the coupling interaction between Arg492 and Asp210 (0.3 kcal/mol, Figure 5 panel C vs. D). As expected from the large dependence of the observed cooperativity between Tyr497 and Asp206 on Arg492, the presence of Arg492 is also critical for the favorable coupling interaction between Tyr497 and Asp210. Eliminating Arg492 results in a negative value of -0.7 kcal/mol for the interaction of Tyr497 and Asp210 (Figure 5 panel E vs. F). Again, as for the interactions with Asp206, the ternary coupling of these three residues keeps the overall interaction highly cooperative (~ 1.9 kcal/mol). Taken together, these data imply that the interactions between Tyr497 and the rest of hotspot residues are largely influenced by the network of pre-established neighboring interactions centered around Arg492 on Cdc25B and Asp206 on Cdk2.

DISCUSSION

Elucidating the energetics of protein-protein interactions has been the subject of many previous experimental and theoretical studies. The most important discovery thus far has been the determination that only a few interfacial residues, the hotspot residues, contribute the majority of binding energy to any given protein-protein interaction¹. The extensibility of hotspot theory has been demonstrated in a wide variety of systems ranging from antibody-antigen complexes, to proteases and their protein inhibitors, to protein hormones and their receptors (reviewed in³). The identification of hotspot residues is in itself a difficult problem that has been investigated by multiple computational and experimental methods. Beyond their mere identification, however, the determination of how hotspot residues contribute to protein-protein interactions is a question of at least equal interest. Experimentally, mutant cycle analyses have proven to be the most powerful method for quantitating the roles of specific interactions at protein interfaces. For example, double mutant cycle experiments for barnase bound to barstar², antibody bound to lysozyme^{24; 25}, Ras bound to its effectors²⁶, and antibody bound to antibody²⁷ have demonstrated that individual buried salt bridges and hydrogen bonds contribute 0.8 - 6 kcal/mol toward protein binding.

Our model system, the Cdc25B – Cdk2-pTpY/CycA interaction, manifests several striking differences to other protein-protein pairs to which mutant cycle analysis has been applied. First, Cdk2-pTpY/CycA is a substrate of Cdc25B, as opposed to an unchanging binding partner. Thus, binding is not the end goal, but rather a prelude to catalysis. Second, the affinity between Cdk2-pTpY/CycA is comparatively weak compared to these previously characterized protein pairs. The nM affinity in these other complexes is driven primarily by slow rates of dissociation ($k_{\text{off}} < 0.001 \text{ s}^{-1}$). For Cdc25B, the K_m describing substrate binding and catalysis is $0.4 \mu\text{M}$ ($\neq K_d$) and efficient substrate recognition is driven by a fast rate of association ($k_{\text{on}} = 10^6 \text{ M}^{-1}\text{s}^{-1}$, manuscript in preparation). Despite these differences, the interaction energies we have reported herein for the cluster of hotspot residues at the Cdc25B – Cdk2-pTpY/CycA interface (0.7 – 3.9 kcal/mol) are in good agreement with these previous reports. Additionally it should be noted that as for other complexes, Ras and its effectors in particular²⁶, the majority of the coupling energies for our system can be accounted for by charged residues.

However, as previously noted by others⁸, pair-wise interactions can be deceiving as interaction energies are often cooperative in nature. In fact, neutral or negative contributions of salt bridges

have been demonstrated experimentally for TEM-1- β -lactamase and its inhibitor BLIP¹⁵. By converting the reference state to all-Ala, wherein the entire cluster of hotspot residues was mutated away, single pair-wise interactions could be evaluated in the presence or absence of other residues so as to probe the network of coupling energies more thoroughly. For the HyHEL-10 – lysozyme interaction, a thermodynamically insignificant salt bridge has been noted wherein the original Asp32-Lys97 pair can be effectively replaced with an Asn-Met ($\Delta\Delta G_{\text{int}} = 0.3$ kcal/mol) but not an Ala/Ala pair ($\Delta\Delta G_{\text{int}} = 4.6$ kcal/mol)²⁴. Data such as these have supported the viewpoint that in most cases individual salt bridges do not contribute significantly to binding because of the large entropic penalty associated with prior desolvation²⁸.

To as best as possible understand the complex energetics of our transient protein-protein interactions, we have herein probed the roles by which three hotspot residues on Cdc25B (Arg488, Arg492, Tyr497) interact with two hotspot residues in the Cdk2-pTpY/CycA substrate (Asp206, Asp210) using both multiple amino acid substitutions and double and triple mutant cycles. Although one might argue that individual or multiple mutations can disrupt the integrity of our proteins via non-local conformational rearrangements, our control experiments exploit alternative functions (Cdc25 phosphatase activity with small molecules, Cdk2/CycA kinase activity with histones) as probes against such non-specific effects. The self-consistency we observe throughout our data lends credence to the roles we describe for each of the three hotspot residues on Cdc25B:

The role of Arg492

In the docked model of Cdc25B – Cdk2-pTpY/CycA, Arg492 appears to be the key residue for the hotspot residues in this interaction site remote from the active site (Figure 1). Arg492 forms a bidentate hydrogen bond with Asp206 and a possible water mediated hydrogen bond with Asp210²¹. This central salt bridge is reminiscent of the Arg83 – Asp39 interaction in barnase-barstar², although the double mutant cycle coupling energy is somewhat lower (3.9 vs. 6.1 kcal/mol). Our data are most consistent with the interpretation that the majority of the observed cooperativity between Arg492 and Asp206/Asp210 originates from direct pair-wise interactions. These data include the single mutant R492K having less activity than R492L, the double mutant cycle wherein R492K cannot restore coupling energy with Asp206, and the triple mutant cycle wherein significant cooperativity is seen independent of Tyr497. Specifically regarding this latter point, although the presence of Tyr497 does significantly enhance the cooperativity between these two energetically important hotspot residues, we do not observe negative coupling energies between Arg492 and either of the two Asp residues in the absence of Tyr497. The direct pair-wise interactions between Arg492 and Asp206/Asp210 thus appear to be more independent than the electrostatic network in β -lactamase and its protein inhibitor BLIP where negative coupling energies were observed between charged residues¹⁵. Alternatively, since we did not start our triple mutant analysis with an all-Ala background, it is possible that Arg492 establishes its salt bridge network through cooperativity with Arg488. Such higher order interactions have not been measurable as the double mutation of Arg488 and Arg492 severely disrupts the overall stability of Cdc25B.

The role of Tyr497

We propose that the role of Tyr497 is to cooperatively stabilize the energetic network centered around Arg492 and Asp206, which is the most pivotal contact between Cdc25B and its protein substrate. Both the hydrophobic (aromatic ring) and hydrophilic (hydroxyl) components of Tyr497 are critical for maximizing the interaction between Arg492 and Asp206, whereas retaining a competent hydrogen bond network is sufficient for stabilizing the energetically less important interaction between Arg492 and Asp210. Such a dual role for Tyr at protein-protein interfaces has been experimentally observed by double mutant cycles for HyHEL-10 –

lysozyme wherein all Y->A mutations showed a larger $\Delta\Delta G_{\text{int}}$ than the corresponding Y->F mutations. Tyr appears to be ideally suited for forming both polar and apolar interactions at a protein interface.

We summarize the accumulated data supporting this role for Tyr497: First, although the $k_{\text{cat}}/K_{\text{m}}$ of Y497A towards protein substrate was essentially identical to the R492L mutant, the observed coupling energies between Tyr497 and the two Asp residues were much smaller than for Arg492 (also compared to Arg488). It is unlikely that we failed to identify an alternative direct interaction partner for Tyr497 on Cdk2, as mutation of four neighboring residues on Cdk2 did not perturb the reaction with wild-type Cdc25B²¹. Second, the observed cooperativity between Tyr497 and Arg492 on Cdc25B (2.1 kcal/mol) was higher than that seen between Tyr497 and the two Asp residues on Cdk2 (both ~ 1.5 kcal/mol). This pairwise coupling energy between Arg492 and Tyr497 is in fact the highest observed value from all the double mutant cycles involving Tyr497. Third, the observed $k_{\text{cat}}/K_{\text{m}}$ for R492K is marginally lower than that seen for the R492L-Y497A double mutant (300 vs. 800 $\text{M}^{-1}\text{s}^{-1}$), indicating that a single unfavorable interaction at position 492 can account for the loss of both Arg492 and Tyr497. Fourth, in our double mutant cycles with alternative substitutions, each component of Tyr497 seemed to indirectly involve the cooperative interaction of the network centered around Arg492 and Asp206 (Figure 2). Finally, the analysis by triple mutant cycle sheds further light on these data, demonstrating that the interaction between Arg492 and Asp206 (Asp210) is largely responsible for the observed cooperativity between Arg492 and Tyr497 and more significantly for the cooperativity between Tyr497 and Asp206 (Asp210).

Structurally, Tyr497 forms a π -cation interactions with Arg492 within the same α -helix (Figure 1), which is thought to contribute ~ 4 kcal/mol of stabilization energy²⁹. The loss of this internal interaction may be costly for the energetics of the interfacial interactions. For example, the elimination of Tyr497 may perturb the position of Arg492, thus disrupting its interactions with Asp206 and Asp210. A small deviation in such a specific ionic interaction (~ 0.1 Å) can be surprisingly costly (~ 1.3 kcal/mol)^{30; 31}. Alternatively, the removal of Tyr497 located at the outer edge of the interface may expose the bi-dentate hydrogen bond between Arg492 and Asp206 to solvent, thereby forcing it to compete with 55 M water. Recall however, that eliminating Tyr497 does not completely disrupt the ionic network centered around Arg492-Asp206.

The role of Arg488

The positive charge provided by Arg488 is likely the most important functional contribution of this residue in the hotspot network, consistent with the docked model of Cdc25B with Cdk2-pTpY/CycA wherein Arg488 makes only one ionic hydrogen bond with Asp206 (Figure 1). Our evidence includes the significant restoration of activity and change in coupling energy of R488K compared to R492L or R492A.

Concluding remark: Structure-activity relationships

Although highly informative, structural data alone are unable to reveal energetics of hotspot residues at an interface of two interacting proteins. Therefore, to understand how two proteins come together to form a functional complex, detailed biochemical activity measurements as described herein are required. The combination of solid structure-function data can then be used to address the biology of the system under study. For example, having identified and characterized the hotspot residues on Cdc25 and validated their importance in vivo, we have set a foundation for the rational design of small molecule inhibitor that can specifically target this interaction and potentially serve as lead molecules for studying the biology of Cdc25 phosphatases inside the cell.

Materials and Methods

Mutagenesis and Protein Expression

Each mutant construct of Cdc25B and Cdk2 was prepared using the QuickChange protocol from Stratagene. The catalytic domain of Cdc25B (wild-type and mutants) were expressed as untagged proteins following isopropyl β -D-thiogalactoside induction in *Escherichia coli* BL-21(DE3) as described previously²². Mutations were confirmed by DNA sequencing and matrix-assisted laser desorption ionization (MALDI) mass-spectrometry with post source decay (PSD) sequencing of the purified proteins. The Cdk2/CycA complex, in which the CyclinA is truncated and encompasses residues 174-432, was prepared as previously described^{19; 32}.

Enzymatic Assay

All phosphatase reactions were performed in a three-component (3C) buffer system (50 mM Tris, 50 mM Bis-Tris, 100 mM Na acetate, pH 6.0-6.5) containing 2 mM DTT at 25°C. Reactions using *p*-nitrophenyl phosphate (pNPP) and 3-*O*-methyl fluorescein phosphate (mFP) were followed by continuous UV-Vis spectroscopy at 410 nm ($\epsilon = 5142 \text{ M}^{-1}\text{cm}^{-1}$, at pH 6.5) and 477 nm ($\epsilon = 27,200 \text{ M}^{-1}\text{cm}^{-1}$), respectively. All assays using pNPP and mFP consisted of complete K_m determinations using 7 concentrations of substrate between 0.2 and $5 \times K_m$. The bisphosphorylated Cdk2-pTpY/CycA substrate was prepared by incubation of the GST-tagged Myt1 kinase bound to GSH-Sepharose beads and 2.5mM [γ -³²P]-ATP (200-500Ci/mol) for 2.5 h at RT. After removing the unincorporated ATP and Myt1 by G-50 chromatography, Cdk2-pTpY/CycA could be assayed with Cdc25 by monitoring release of inorganic phosphate. Reactions were initiated by enzyme in the presence of 1 mg/ml BSA and 0.05% Tween 20, and quenched and precipitated by adding 3-5 volumes of 30% trichloroacetic acid (TCA). After centrifugation at 14000 X g for 20 min at 4°C to remove the unreacted substrate, the supernatant was subjected to scintillation counting. For k_{cat}/K_m measurements, time-dependence (5 points minimum), substrate concentration dependence (2 - 3 differing concentrations), and enzyme concentration dependence (4-5 differing concentrations) were tested in minimum of duplicate to ensure Michaelis-Menten conditions.

Mutant cycle analysis

Differences in k_{cat}/K_m s between mutants for the double mutant cycles were converted to the change in free energy using

$$\Delta\Delta G_I = -RT \times \ln \left[\frac{(k_{cat}/K_m)_{mut}}{(k_{cat}/K_m)_{WT}} \right] \quad (\text{Equation 1})$$

wherein R is the gas constant and T is the temperature in degree Kelvin. The coupling energy ($\Delta\Delta G_{int}$) is then derived by constructing a thermodynamic box (or cube) (Figure 3), and can be described by the following equation:

$$\Delta\Delta G_{int} = (\Delta\Delta G_1 + \Delta\Delta G_2) - \Delta\Delta G_{12} \quad (\text{Equation 2})$$

wherein the $\Delta\Delta G$ s reflect the free energy changes for the two mutants together or separately as noted by the subscripts. Error propagation for the double mutant cycles was performed as described²⁴ and for the $\Delta\Delta G_{int}$ is less than 0.7 kcal/mol.

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Abbreviations

Cdc25, Cell division control 25; Cdk2, Cyclin dependent kinase 2; Cdk/CycA, Cdk2 complexed with cyclin A; Cdk2-pTpY/CycA, Cdk2/CycA that is bis-phosphorylated on Thr14 and Tyr15 by the Myt1 kinase; DTT, dithiothreitol; GSH, glutathione; mFP, 3-*O*-methylfluorescein phosphate; pNPP, *p*-nitrophenyl phosphate; 3-C buffer, 3-component buffer; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry.

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Figure 1. Snapshot of the Cdc25B – Cdk2-pTpY/CycA complex highlighting the network of hotspot residues at the remote docking site. Cdc25B, Cdk2, and CycA are shown in cartoon in purple, blue, and gray, respectively. The active site region is denoted by a dashed circle and pThr14 and pTyr15 of Cdk2 and the active site loop of Cdc25 (Cys473 to Arg478) are shown in licorice. The remote hotspot is denoted by a dotted circle, with a blow-up on the right showing the average distances (heavy atom to heavy atom) observed from the molecular dynamics trajectory at 305K from 200 – 1000 ps²¹. Residues from Cdk2 are underlined.

Arg488

D206A	2.9	2.8	1.1	-	
D210A	1.4	1.6	0.8	-	
Arg492

D206A	3.5	3.9	3.1	-	
D210A	1.5	1.8	1.6	-	
Tyr497

D206A	1.6	2.3	0.7	-	
D210A	1.5	1.9	1.3	-	

Figure 2.

Double mutant cycle analysis with alternative hotspot mutants. The pair-wise coupling energies ($\Delta\Delta G_{\text{int}}$) were determined according to Equation 1 and 2. The listed numbers are mean values of at least three separate determinations (± 0.7) and in units of kcal/mol.

Figure 3.

Construction of a cube for triple mutant cycle analysis.

MT_i represents each mutant residue whereas WT denotes a native residue. Each face of the cube represents a complete double mutant cycle, and opposing faces are identical except one cycle includes the third mutant residue. The difference between $\Delta\Delta G_{int}$ s obtained from the opposing faces of cube represents the ternary coupling energy of the entire system.

Figure 4.

Calculations of pair-wise coupling energies for triple mutant cycle analyses of R492L/Y497A/D206A.

Two horizontally juxtaposed boxes are opposing faces of the triple mutant cycle cube described in Figure 3. Each three-digit number represents the absence of the particular wild-type residue (i.e. R492L, Y497A, D206A) whereas WT stands for the presence of the native residue. Each value next to an arrow represents the observed (normal text) or calculated (italicized text) change in free energy upon mutation. The $\Delta\Delta G_{int}$ and the set of three WT and mutant residues in the center of each cycle stands for the determined coupling energy for the two mutant residues in the presence or absence of the third residue (panel A vs. B, etc.). A positive $\Delta\Delta G_{int}$ represents a favorable pair-wise interaction, a negative value represents an unfavorable interaction, and zero is neutral. The difference of $\Delta\Delta G_{int}$ s between two side-by-side boxes is the ternary coupling energy and is identical for all three pairs of double mutant cycles. All the values are in units of kcal/mol.

Figure 5. Calculations of pair-wise coupling energies for triple mutant cycle analyses of R492L/Y497A/D210A. As for Figure 4, but replacing D206A with D210A.

Summary of kinetic parameters of wild-type and hotspot mutants of Cdc25B. Wild-type and the original hotspot mutant data (R488L, R492L, and Y497A) are taken from reference 21.

Table 1

Cdc25B	pNPP k_{cat} (s^{-1})	pNPP k_{cat}/K_m ($M^{-1}s^{-1}$)	mFP k_{cat} (s^{-1})	mFP k_{cat}/K_m ($\times 1000 M^{-1}s^{-1}$)	Cdk2-pTpY /CycA k_{cat}/K_m ($\times 1000 M^{-1}s^{-1}$)
WT	0.08 ± 0.02	13 ± 2	0.7 ± 0.2	83 ± 18	856 ± 180
R488A	0.18 ± 0.01	32 ± 3	0.5 ± 0.1	100 ± 20	1.1 ± 0.4
R488L	0.11 ± 0.02	20 ± 4	0.7 ± 0.2	56 ± 10	1.3 ± 0.3
R488K	0.18 ± 0.01	32 ± 3	0.5 ± 0.1	100 ± 15	47 ± 3
R492A	0.10 ± 0.01	10 ± 2	0.3 ± 0.2	48 ± 7	20 ± 5
R492L	0.11 ± 0.01	10 ± 1	0.3 ± 0.3	48 ± 6	3.3 ± 0.5
R492K	0.12 ± 0.05	19 ± 2	0.9 ± 0.2	210 ± 20	0.29 ± 0.02
Y497A	0.12 ± 0.04	15 ± 1	0.8 ± 0.1	68 ± 2	4.1 ± 0.7
Y497T	0.20 ± 0.01	34 ± 6	0.7 ± 0.1	86 ± 15	2.5 ± 0.6
Y497F	0.11 ± 0.02	16 ± 3	0.7 ± 0.1	46 ± 9	100 ± 20
R492L-Y497A	0.13 ± 0.03	17 ± 2	1.2 ± 0.4	260 ± 50	0.8 ± 0.1

Table 2

Pair-wise coupling energies in the presence or absence of adjacent residues as derived from triple mutant cycle analyses. Data are summarized from Figures 4 and 5 and all values are in units of kcal/mol.

other mutations	Arg492 – Tyr497	Arg492 – Asp206	Tyr497 – Asp206	Arg492 – Asp210	Tyr497 – Asp210
none	2.1	3.9	1.6	2.2	1.3
R492L			-1.3		-0.7
Y497A		1.1		0.3	
D206A	-0.8				
D210A	0.2				